Proc. Natl. Acad. Sci. USA
Vol. 77, No. 9, pp. 5542–5546, September 1980
Neurobiology

The differential effects of ionizing radiation on the circadian oscillator and other functions in the eye of *Aplysia*

(x-rays/molluskan eye)

JOHN C. WOOLUM* AND FELIX STRUMWASSER†

Division of Biology, California Institute of Technology, Pasadena, California 91125

Communicated by James F. Bonner, June 9, 1980

ABSTRACT **Ionizing radiation has been used to selectively separate the circadian oscillator function of the eye of *Aplysia* from some of its other functions—synchronous compound action potential (CAP) generation, the light response, synaptic transmission between photoreceptors and output neurons, and the bursting pacemaker mechanism. Doses of 4-krad (50 kV peak) x-rays have a minimal effect on the circadian rhythm of CAP frequency, measured from the optic nerve, whereas irradiation with a 40-krad dose abolishes the rhythm without affecting any of the four other functions of this eye (1 rad = 0.01 J/kg = 0.01/Gy). We estimate a 50% survival of the oscillator function at doses of about 6 krad. The oscillators of irradiated eyes are not merely desynchronized when the rhythm is abolished, because *in vitro* light–dark entrainment does not restore free-running rhythmicity. The results, including those from selective irradiation of the anterior or posterior poles of the eye, suggest that there are a number of circadian oscillators in the eye—most of them in the posterior portion near the optic nerve. An approximate target size has been obtained from target theory, $\approx 10^8$ Å^3, which is somewhat larger than the target size for viral infectivity function, as one example. There are reservations about estimating target size in a complex organ such as the eye. However, this approximate target size and the fact that recovery or repair can occur *in vivo* suggest that the oscillator may involve nucleic acid molecules.**

A number of recent studies have indicated that circadian oscillators can be localized to certain small portions of the nervous system [suprachiasmatic nuclei in mammals (1, 2); pineal organ in birds (3, 4)], but there is little information on how such circadian oscillators are organized in terms of cellular events and interactions. The isolated eye of *Aplysia* contains a circadian oscillator because the frequency of optic nerve action potentials cycles with an approximately 24-hr period, even though the eyes are maintained in constant darkness (5). Isolated eyes maintained in a simple ionic medium, sea water, will generally cycle four to six times with a period of 23.4 ± 0.3 hr (SEM) at 14°C (6). The eye contains at least three neuronal cell types besides photoreceptors (7). It is not known which, if any, of these cell types generates the rhythm or whether interactions between cells are required for the genesis of the long period.

We thought that ionizing radiation could perhaps be used as a tool to dissect the cellular mechanisms of the circadian rhythm, as has been done in dissecting viral functions (8). Ideally, a specialized process carried out by certain cells might be more sensitive to x-ray damage than other processes in the same or other cells. The eye is, of course, specialized to transduce light into an electrical signal. Another specialization of the eye of *Aplysia* is to mediate by coupling between cells both spontaneous and light-evoked discharges, as recorded from the optic

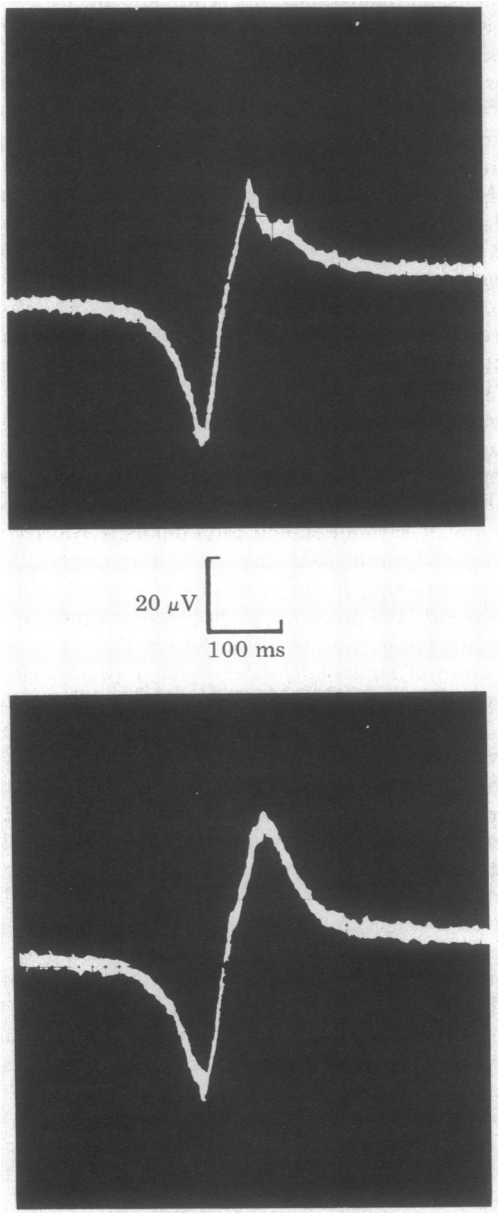

FIG. 1. Wave shapes at 22°C of spontaneous CAPs in the optic nerve of *Aplysia* eye after adapting to dark for 30 min and just before a 40-krad dose of x-rays (*Upper*) and 80 min after x-rays (*Lower*).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviation: CAP, compound action potential.

* Permanent address: Dept. of Physics, California State University, Los Angeles, CA 90032.
† To whom reprint requests should be addressed.

nerve, because each action potential is normally a compound spike (9). Gap junctions have been found between photoreceptors (10) and between neurosecretory cells (7), and either or both of these specializations could mediate the normal synchrony of neuronal discharge during a compound action potential (CAP).

Surprisingly, our studies show that the circadian mechanism in the isolated eye of *Aplysia* is quite sensitive to x-rays. Four other specialized cellular processes in the eye—phototransduction, synaptic transmission, the coupling mechanism mediating CAP synchrony, and the bursting pacemaker mechanism—are relatively insensitive to x-rays, because they remain intact under conditions that clearly affect the circadian oscillator. We also find that a larger effect on the oscillator is obtained when the posterior part of the eye is irradiated than when the anterior part is irradiated. In addition, we report here that damage to the circadian oscillator by irradiation of the eye *in vivo* appears to be repairable, at least by 20 days.

METHODS

Animals (*Aplysia californica*) used in these experiments were maintained on a 12-hr light, 12-hr dark cycle for at least 4 days before dissection. Eyes were dissected at about 2 hr after lights were on and were maintained in Millipore-filtered (0.22 μm) sea water during irradiation. Irradiation was done with 50 kV peak x-rays from a Machlett OEG 60 tube with a tungsten target, beryllium end window, and 0.25-mm aluminum filter. A dose rate of 4 krads/min (1 rad = 0.01 J/kg = 0.01 Gy; measured with a Victoreen rate meter) was obtained under these conditions at a distance of 4 cm from the end of the tube at a beam current of 20 mA (the doses over 100 krad were given at a distance of 1.5 cm, where the dose rate was 8 krad/min). Eyes along with their optic nerves were irradiated in dim light conditions (3 lux) in a Sylgard dish in which the fluid level had been lowered to just cover the eye. More than 80 eyes were given doses between 2 and 480 krads in this way.

In some experiments a 1.0-mm thick lead shield was placed over part of the eye in order to irradiate only the unshielded part of the eye. To determine if the unshielded part of the eye was receiving the full dose expected, a microdensitometer trace of x-ray film was run near an area of the film that had been shielded by a lead shield during x-ray exposure. The x-ray intensity was 80% of maximum within 100 μm of the edge of the shield, so the dose received varied only over about 100 μm. For an eye 800 μm in diameter, at least a 300-μm portion received greater than 80% of the dose if half the eye was covered by the shield. A 1-mm lead shield decreased the dose of x-rays of 50 kV peak energy by a factor of more than 100. For *in vivo* irradiation, the animal was first anesthetized with 50% isotonic Mg^{2+} by immersion in the modified sea water for 2 hr. At the end of this period, the animal was covered with 2 mm of lead and the skin on each side of the eye was pulled with forceps through a 6-mm hole in the lead. The eye was then irradiated in this position.

Measurements of eye CAPs during circadian rhythm recordings were made in a light-tight box. The optic nerves were held in suction electrodes with the eyes placed in 35-mm tissue culture dishes containing about 5 ml of minimal essential medium with 15–30 mM bicarbonate in filtered sea water at pH 7.7. This medium contains all 20 amino acids at concentrations used with mammalian cell cultures (11). It has been shown to maintain normal resting potentials, action potentials, and synaptic mechanisms in organ-cultured ganglia of *Aplysia* (12).

RESULTS

In order to evaluate the effects of x-rays on cellular processes in the eye other than the circadian rhythm, we compared four functions before and after x-irradiation. The waveshape and amplitude of the optic nerve CAP is a measure of the number and degree of synchrony or coupling of discharging cells in the population of output cells of the eye, provided that the recording conditions remain unchanged. When the eye becomes active, the pattern of CAPs occurs in groups of two to six with longer silent intervals in between these groups of CAPs. We refer to this pattern as a bursting pacemaker mechanism, because it is similar to the burst of impulses followed by a longer interval of silence seen in endogenously active neurons of the abdominal ganglion (13, 14). The third and fourth functions that we assayed were phototransduction in photoreceptor cells and synaptic transmission between the receptors and the output cells as recorded by CAPs in the optic nerve. Audesirk (15) has provided evidence, from experiments with propionate substituted for chloride, that receptors are electrically coupled to the output cells. Evidence for the presence of gap junctions between photoreceptors and between these and other neuronal cells exists (7, 10).

Fig. 1 compares the CAPs from the same eye before and after irradiation (40 krad). The CAP amplitude and waveshape was not altered by the x-rays. Fig. 2 shows the bursting pattern of CAPs of two eyes obtained from the same animal; the control eye and irradiated eye (40 krad to the whole eye) are compared 3 days after irradiation. The ability of the eye to produce the normal bursting pattern of CAPs is unaffected by x-rays. The

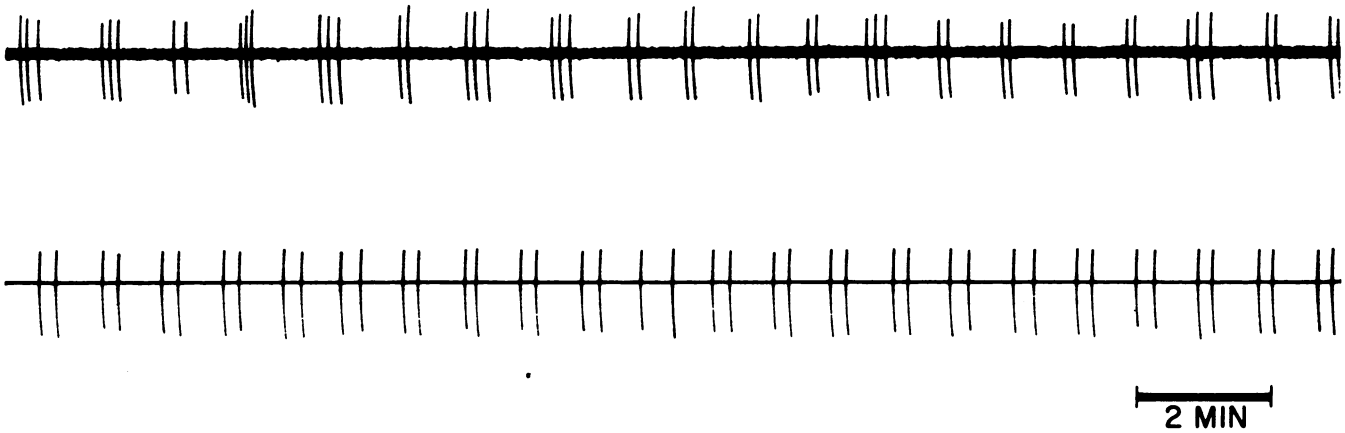

FIG. 2. Bursting pattern of spontaneous CAPs from irradiated eye (40 krad, *Upper*) and control eye (*Lower*) 3.5 days after irradiation. Eyes were in continuous dark. t = 13°C for this and subsequent figures.

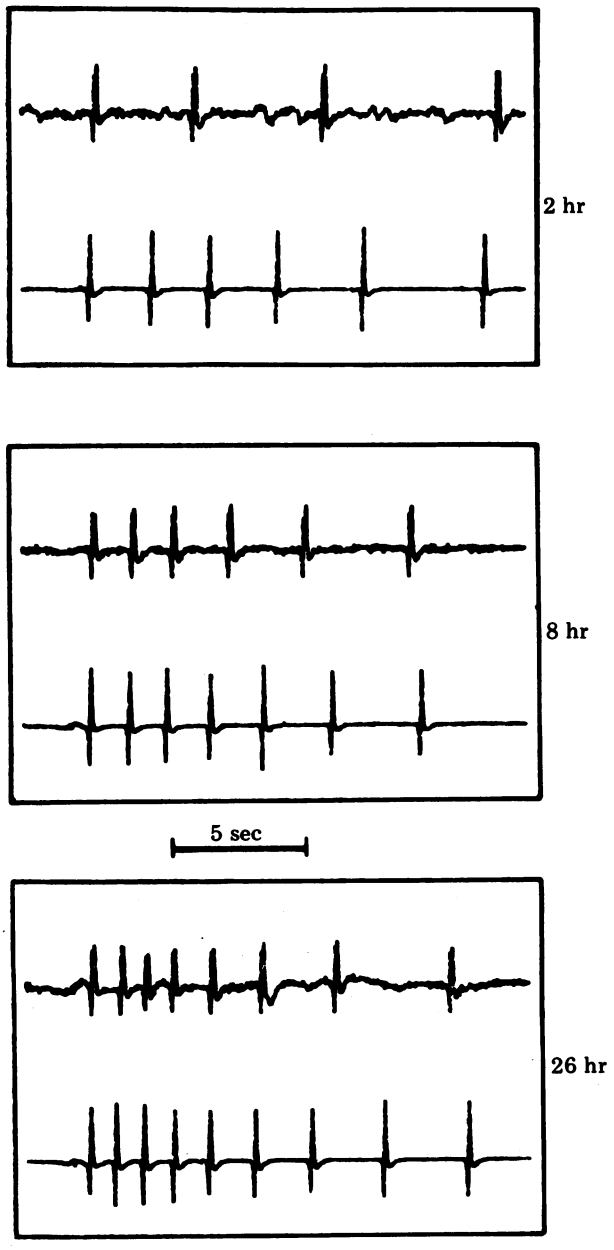

FIG. 3. Comparison of light-evoked CAPs in irradiated (40 krad) and control eyes. Eyes were obtained from the same animal and the times shown represent hours after x-irradiation. In each frame, the top line is CAPs from the irradiated eye and the bottom line is CAPs from the control eye. Light onset is 0.13 sec after the start of each sweep, and light intensity was fixed at about 0.1 lux.

variation in burst size of the irradiated eye, evident in Fig. 2, was also observed in control eyes. Fig. 3 shows the response to light (0.1 lux) of a control eye and an irradiated eye (40 krad to the whole eye) at 2 hr, 8 hr, and 26 hr after irradiation. The light response of the x-irradiated eye was normal. Doses of up to 480 krad (in other experiments) did not appreciably affect the size and shape of the CAP, the bursting pattern, or the light response.

Fig. 4*A* shows the CAP rate for 4 days from a control eye and from an irradiated eye (40 krad to the whole eye). It can be seen that 40 krad completely stopped the expression of the circadian rhythm. In some cases, as shown here, there appeared to be a partial remnant of the rhythm for the first cycle. Fig. 4*B* shows the average CAP frequencies of six or seven eyes at five different doses (0–40 krad) beginning 6 hr before the projected dawn and about 15 hr after irradiation with x-rays. The amplitude of the circadian rhythm decreased with increasing dose. The effect of a 4-krad dose on the rhythm ($n = 4$) was to decrease the amplitude less than 15% relative to the paired controls ($n = 2$) and was therefore minimal. Though there was some phase delay of the rhythm for eyes that received 8 krad (not shown), the period was very close to the control [about 26 hr, which is normal in the enriched medium used in these experiments (16)]. The lower doses (typically greater than 80 krad) increased the average CAP rate after an initial slow period (e.g., Fig. 4*A*).

Fig. 5 shows the CAP rate for two eyes irradiated with 60 krad with a lead shield over part of the eye. The curve with filled squares is for an eye with the shield over the optic nerve pole of the eye (anterior part irradiated) and the curve with open circles is for an eye with the shield over the anterior part of the eye (posterior part irradiated). The amplitude of the circadian rhythm was greatly reduced (totally in some eyes) when the posterior part of the eye was irradiated but reduced only slightly when the anterior part was irradiated. This result is in agreement with experiments that report the presence of a circadian rhythm in eyes trimmed to the extreme base (17).

Eyes dissected from animals that were irradiated *in vivo* (40 krad) had no rhythm if they were dissected on the same day that they were irradiated ($n = 2$). However, if the animals were dissected 20 days or more after irradiation ($n = 5$), the eyes had a normal rhythm. These results suggest that there is some form of *in vivo* recovery or repair over long times. No *in vitro* repair was observed in periods as long as 5 days.

DISCUSSION

The doses of x-rays used here seem to be quite large but are typical of what nerve tissue can easily tolerate. For example giant nerve fibers from the earthworm, when continuously irradiated at 6 krad/min with 280 kV peak x-rays, show increased spike amplitude and conduction velocity at doses up to 78 krad but show conduction blockage at doses over 240 krad (18). *Aplysia* pacemaker neurons of the abdominal ganglion are activated by doses of a few kilorads of 20 MeV electrons but are inactivated by about 200 krad and are not able to recover after such large doses, at least within 8 hr (19). Our results do not confirm this for other neurons in *Aplysia* since in irradiated eyes, at doses of over 200 krad (240, 320, and 480 krad), we find that spontaneous CAP frequency is decreased for about 1 day and then increased for several days. It is rather fortunate that the circadian oscillator is much more sensitive to ionizing radiation than are the four other functions of the eye measured in this work.

The simplest interpretation of the effect of x-rays on the circadian rate of CAPs in the optic nerve is to assume that there are a number of circadian oscillators distributed throughout the eye (mostly in the posterior part, see Fig. 5) and that the amplitude of the circadian rhythm is proportional to the number of circadian oscillators that are active and in phase. When all the circadian oscillators in either the anterior or posterior parts of the eye (but not in both parts) or when only some of the circadian oscillators in the entire eye (small dose) are hit, the remaining circadian oscillators produce a rhythm of reduced amplitude. It appears that the x-rays do not just desynchronize the circadian oscillators, because we have been unable to restore the CAP rhythm to an irradiated eye by putting it in 12 hr of light (10 lux) and 12 hr of dark for 3 days and recording frequencies from it for at least 3 more days ($n = 2$).

An alternative interpretation of the blocking effect of x-rays on the circadian oscillation is that the oscillator remains intact

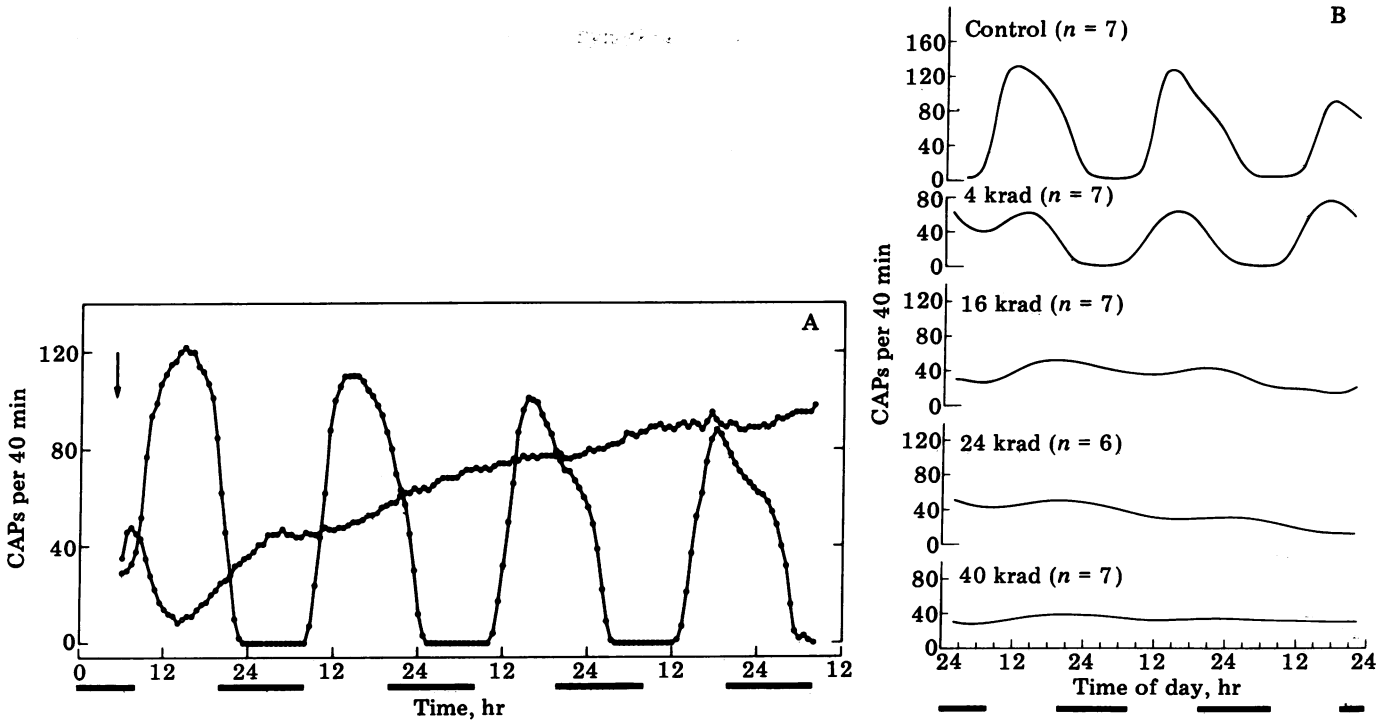

FIG. 4. (*A*) CAP frequencies for an eye which received 40 krad (downward arrow) about 2 hr before dawn (O—O) and an unirradiated eye from the same animal (●—●). Rectangular bars below time-of-day axis indicate the projected dark–light schedule that the intact animal had received. (*B*) Average CAP frequencies from four to seven eyes that received one of four doses between 4 and 40 krad 15 hr before the start of these records. The control is shown at the top.

but that its expression is blocked. The expression of the oscillator could depend on an x-ray-sensitive coupling mechanism between the oscillator and membrane, for example. We do not favor this alternative because, as discussed later, we find absolutely no evidence for any interference with the membrane functions that can be assayed in the eye by extracellular recordings. We also have tested x-ray sensitivity in one other neural system in *Aplysia* for which there is evidence of mediation by an intracellular messenger—bag cell afterdischarge (unpublished data). Three out of three abdominal ganglion preparations that received 40 krad showed normal bag cell afterdischarge in each of the two clusters per ganglion, lasting 30–45 min after brief electrical stimulation of one pleurovisceral connective, a synaptic pathway in this system. Because afterdischarge is correlated with a rise in cyclic AMP (20) and because 8-benzylthio cyclic AMP induces afterdischarge in bag cells (20), the lack of x-ray sensitivity to 40 krad strongly suggests that this intracellular messenger system is relatively resistant to x-rays. Another intracellular messenger system that is quite insensitive to x-rays is the calcium–sarcoplasmic reticulum system in muscle. Doses of x- or γ-rays greater than 200 krad are needed for inactivation of contraction of gastrocnemius muscle from a number of species (21). Therefore, we feel that the available evidence does not support the possibility that the oscillator is intact and running but that it is uncoupled from the membrane functions that we have measured.

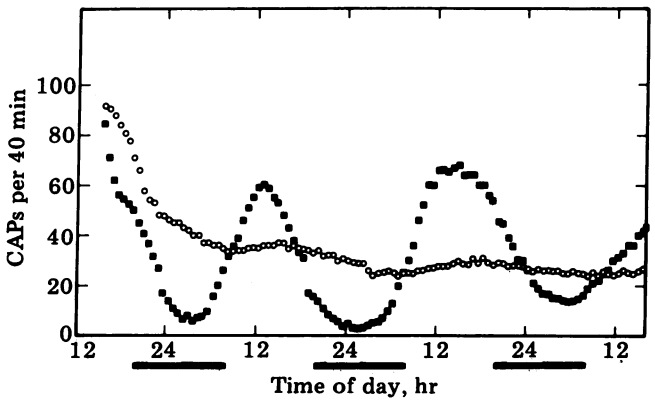

FIG. 5. Cap frequencies from two eyes which received 60 krad to either the anterior (■) or posterior (O) part of the eye (see *Methods* for details).

Ionizing radiation has been used to determine the effective target size associated with a particular function of a virus and, in some cases, the number of such targets per virus. In metabolizing systems, target size is not easily determined because of complications of free radicals and repair of damage (22). Multitarget theory says that the probability of survival of a function is $P_s = 1 - (1 - e^{-IV})^n$, in which I is the number of primary ionizing events per volume (we assume 100 eV are required for an event, because it is known that ≈35 eV will produce one ion–electron pair in a variety of materials), V is the target volume of the function, and n is the number of targets that must be hit in order to stop the function (23). Using 6 krad as an approximate dose for 50% survival of the oscillator function, we compute the target volume to be a few times 10^8 Å^3, a value within one or two orders of magnitude of the target volume of a variety of virus functions (8). This target volume is not very sensitive to the number of targets assumed. Repair to the circadian oscillator would make the correct volume larger than this estimate, and damage done by diffusing agents such as free radicals would make the correct volume smaller. Our results show little or no recovery or repair of the circadian function *in vitro* on the time scale of our experiments. The effects of free radicals are much more difficult to estimate, so this target volume is to be considered only very approximate but perhaps useful as an upper limit.

In the more than 80 eyes irradiated with doses of 2–480 krad,

there was no evidence of an ultradian rhythm, as judged by time series analysis. Therefore, it seems unlikely that there are a number of coupled oscillators in the eye, each one of which generates an ultradian rhythm. This interpretation has been reported for the alleged ultradian behavior of trimmed eyes by Jacklet and Geronimo (24), but the finding has not been confirmed (17). All the available evidence indicates that irradiation produces a more graded diminution of the circadian rhythm than is possible in cutting experiments on the eye done with scissors.

The four functions of the eye that are relatively insensitive to x-rays are primarily membrane functions—photoreception, electrical synaptic transmission, the bursting pacemaker mechanism, and the degree of coupling of the output cells. This implies that although the membrane is an important input/output channel to the clock [e.g., resetting of the phase of the rhythm by high K^+ (25)], it may not be part of the generator of the circadian oscillator as proposed by others (26). The apparent insensitivity of these membrane processes to x-rays is probably accounted for by the small target size or the large number of targets involved.

The total blocking of the circadian oscillators with 40 krad observed here is similar to results obtained with a 3-hr pulse of actinomycin D (27)—an inhibitor of DNA-dependent RNA synthesis. Because recovery from or repair of ionizing radiation damage is usually thought to be associated with nucleic acid systems, the *in vivo* repair results provide some additional evidence for the involvement of nucleic acids in the circadian oscillator.

We thank Profs. Bonner, Delbrück, and Maniatis for their comments on the manuscript. Mr. Floyd Schlechte produced the average curves (shown in Fig. 4) by computer for which we are grateful. We thank Prof. E. B. Lewis for use of the x-ray facility. The research was supported by grants from the National Institutes of Health (NS 07071, NS 13896) to F.S. and by a short-term grant from the National Aeronautics and Space Administration (NSG 7387).

1. Moore, R. Y. & Eichler, V. B. (1972) *Brain Res.* **42,** 201–205.
2. Stephan, F. K. & Zucker, I. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 1583–1586.
3. Gaston, S. & Menaker, M. (1968) *Science* **160,** 1125–1127.
4. Zimmerman, N. H. & Menaker, M. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 999–1003.
5. Jacklet, J. W. (1969) *Science* **164,** 562–563.
6. Rothman, B. S. & Strumwasser, F. (1976) *J. Gen. Physiol.* **68,** 359–384.
7. Strumwasser, F., Alvarez, R. B., Viele, D. P. & Woolum, J. C. (1979) in *Biological Rhythms and Their Central Mechanism,* eds. Suda, M., Hayashi, O. & Nakagawa, H. (Elsevier/North Holland, Amsterdam), pp. 41–56.
8. Setlow, R. B. & Pollard, E. C. (1962) *Molecular Biophysics* (Addison-Wesley, Reading, MA), pp. 358–366.
9. Jacklet, J. W. (1969) *J. Gen. Physiol.* **53,** 21–42.
10. Strumwasser, F. & Alvarez, R. B. (1974) *Caltech Biology Annual Report* (California Inst. of Technology, Pasadena, CA), pp. 225–226.
11. Eagle, H. (1959) *Science* **130,** 432–437.
12. Strumwasser, F. & Bahr, R. (1966) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **25,** 512.
13. Strumwasser, F. (1965) in *Circadian Clocks,* ed. Aschoff, J. (North Holland, Amsterdam), pp. 442–462.
14. Frazier, W. T., Kandel, E. R., Kupfermann, I., Waziri, R. & Coggeshall, R. E. (1967) *J. Neurophysiol.* **30,** 1288–1351.
15. Audesirk, G. (1973) *Brain Res.* **59,** 229–242.
16. Benson, J. A. & Jacklet, J. W. (1977) *J. Exp. Biol.* **70,** 151–166.
17. Strumwasser, F. (1973) *Physiologist* **16,** 9–42.
18. Bachofer, C. S. & Gautereaux, M. E. (1959) *J. Gen. Physiol.* **42,** 723–735.
19. Carpenter, D. O., Gaubatz, G., Willis, J. A. & Severance, R. (1978) *Radiat. Res.* **76,** 32–47.
20. Kaczmerak, L., Jennings, K. & Strumwasser, F. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 5200–5204.
21. Bachofer, C. S., Gautereaux, M. E. & Kaack, S. M. (1964) in *Response of the Nervous System to Ionizing Radiation,* eds. Haley, T. J. & Snider, R. S. (Little, Brown, Boston), pp. 228–229.
22. Bacq, Z. M. & Alexander, P. (1961) *Fundamentals of Radiation Biology* (Pergamon, New York), pp. 82–91.
23. Casarett, A. P. (1968) *Radiation Biology* (Prentice Hall, Englewood Cliffs, NJ), pp. 141–142.
24. Jacklet, J. W. & Geronimo, J. (1971) *Science* **174,** 299–302.
25. Eskin, A. (1972) *J. Comp. Physiol.* **80,** 353–376.
26. Njus, D., Sulzman, F. M. & Hastings, J. W. (1974) *Nature (London)* **248,** 116–120.
27. Rothman, B. S. & Strumwasser, F. (1977) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36,** 2050–2055.